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Identification and Localization of a Ligand Molecule of *Xenopus* Cortical Granule Lectins

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ABSTRACT—This study aimed to identify the ligand molecule of the cortical granule lectins which participate in the formation of the fertilization (F) layer in *Xenopus laevis*. Comparison of fertilization envelopes (FEs) with and without the F layer by SDS-PAGE showed a 105-kDa glycoprotein (gp 105) only in the former FEs. This glycoprotein was isolated by differential centrifugation and electrophoretical extraction from an F layer extract. Immunoblotting with an antiserum against gp 105 produced staining on the gp 105 in the FEs; the relative amount of gp 105 increased during the hatching period due to the digestion of vitelline envelope components. Staining on westernblots with HRP-conjugated peanut agglutinin suggested that gp 105 contains a small amount of galactosides. With immunoelectron microscopy, gold particles indicating the location of gp 105 were visible on the pre-fertilization (PF) layer of eggs obtained from the pars recta 2 (PR2) and the uterus of the oviduct, but they were few on the F layer of activated eggs. With the PA-CrA-Silver method for detecting carbohydrates, silver particles appeared on the PF layer but not on the main body of the F layer. The binding of gold-conjugated lectins to gp 105 on westernblots showed that gp 105 interacts with the cortical granule lectins. It was concluded from these results that gp 105 is a natural ligand of the lectins, and that it resides in the PF layer and is supplied to eggs at the PR2.

INTRODUCTION

Fertilization in *Xenopus laevis* induces the formation of a fertilization (F) layer in the egg envelopes as well as a hydrolytic event which changes the vitelline envelope (VE) of unfertilized eggs to that (VE*) of fertilized eggs [7, 12, 21]. Subsequently, the fertilization envelope (FE) consists of the VE* and the F layer and it acts as a block to polyspermy [5, 6]. The F layer is formed by the interaction of cortical granule lectins with ligand molecules. The nature of the lectins has been well documented [3, 16, 28, 29] but that of the ligand molecules not. Two sources for the ligand molecules have been proposed, the innermost jelly layer [25] and the pre-fertilization (PF) layer [26, 31].

The jelly layers of *Xenopus* eggs are composed of four morphologically distinct layers [27]. Wyrick *et al.* [25] first demonstrated a precipitation reaction on agar plates between the solubilized jelly of the innermost layer and the lectins. Ligand molecules of the jelly were recently identified immunoelectrophoretically by Birr and Hedrick [2] but have not yet been characterized.

Yoshizaki and Katagiri [31] reported a PF layer lying between the VE and the innermost jelly layer of uterine eggs; it is produced by epithelial cells at the pars recta 2 (PR2) of the oviduct [26]. Its honeycomb structure was shown through quick-freeze, deep-etch electronmicroscopy by Larabell and Chandler [11]. When eggs obtained from the PR2 were activated or treated with lectins, their PF layer became similar to the F layer morphologically and biochemically [31]. Furthermore, the F layer could not be produced in activated eggs when they were deprived of the PF layer [31]. The PF layer, then, seems essential for F layer formation. However, the identity of the ligand molecule in the PF layer was not known. How could this ligand molecule be found? The strategy was to compare FE with and without the F layer electrophoretically and then isolate the molecule from an F layer extract. The result was a 105-kDa glycoprotein (gp 105) which was present in the PF layer and interacted with cortical granule lectins.

MATERIALS AND METHODS

Collection of eggs

South African clawed frogs, *Xenopus laevis*, were purchased from a dealer in Hamamatsu, Japan and reared at 22–24°C. Ovulation was induced by injection of 1,000 IU gonadotropin (Gonatropin, Teikoku Zoki Co.) and a sufficient number of oviductal eggs were obtained 7–10 hr after hormone injection. Uterine eggs were obtained by squeezing females every hour after the start of oviposition. Artificial insemination was performed by the method of Moriya [14]. Developmental stages were determined according to the normal table of Nieuwkoop and Faber [15].

Procedures for isolating gp 105

Uterine eggs obtained from 3 females were placed in 0.05 De Boer solution (DB: 110 mM NaCl, 1.3 mM KCl, 1.3 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.4), artificially activated with a 100 V AC current for 10 sec, and left for 30 min in the solution. Eggs were then dejellied by brief treatment with 20 mM dithiothreitol (DTT) in Ca-free 0.05 DB (adjusted to pH 9.0 with NaOH) and washed extensively with 0.05 DB. The F layer was extracted from these dejellied eggs by treatment with 5 mM EDTA in Ca-free 0.05 DB for 10 min. The F layer extract (ca. 100 ml) was dialyzed against distilled water overnight and concentrated to 5 ml by ultrafiltration. A precipitate appeared during the procedure of concentration; this was subsequently centrifuged at 7,000×*g* for 30 min at 4°C. The resulting pellet was suspended in a solution of 20 mM DTT and 5 mM

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EDTA in Ca-free 0.05 DB (pH 9.0), freeze-thawed and centrifuged at 1,000×g for 15 min. The precipitate was subjected to 7.5% SDS-PAGE. Protein bands were visualized by treatment with 4 M sodium acetate [8]. Sections of 3 mm width at Rf=0.3 were sliced out and cut into several pieces. The gp 105 was extracted electrophoretically from the pieces of gel into 2.5 mM Tris-HCl buffer, pH 8.3, in a Max-Yield Protein Concentrator (Atto Co.) at 5 W for 2.5 hr.

Preparation of egg envelopes and cortical granule lectins

A jelly solution was obtained from activated uterine eggs by DTT-treatment and dialyzed against distilled water. It was then lyophilyzed and dissolved in DB when used.

VE*s were isolated by the method of Wolf *et al.* [24] from eggs whose F layer had been removed. The eggs were crushed by passage through a hypodermic syringe with an 18 G-gauge needle and the VE*s were sieved out through a nylon mesh (82 μm). After thorough washing in distilled water, the VE*s were collected by centrifugation. Before being used, they were suspended in DB with an ultrasonic vibrator.

Egg FEs were isolated from dejellied eggs by the same method as mentioned above. Embryonic FEs were prepared as follows. Embryos were dejellied at stages 17–19 and cultured in 0.05 DB. The FEs were isolated from the embryos manually with watchmaker's forceps. Since dejellied embryos would hatch precociously at stage 28, FEs of later embryos were obtained by incubating the FEs and embryos at stage 28 for 15 hr from the time of reaching stage 28 [32].

Cortical granule lectins were obtained from activated coelomic eggs and purified with an affinity column [29]. The lectins were labeled with colloidal gold prepared by the tannic acid procedure of Slot and Geuze [18]. Lectin-gold complexes were made at 4°C by mixing twice the minimal stabilization amount of lectins with the colloidal gold solution. The mixtures were then centrifuged at 100,000×g for 30 min and the precipitates suspended at an approximate concentration of 20 μg/ml lectin in DB solution containing 1% BSA.

Electrophoresis

Slab SDS-PAGE in 7.5% gel was carried out as described by Laemmli [10]. The gels were stained with Coomassie blue or PAS. The molecular weights of the protein were estimated from a calibration curve obtained with the standard proteins in the MW-SDS-200 kit (Sigma). Protein was determined by the method of Smith *et al.* [20] using bicinchoninic acid with BSA as a standard.

Detection of carbohydrates on westernblots

Electrophoresed samples were electroblotted to nitrocellulose membranes. Galactosides were detected using the method developed by Kitagaki-Ogawa *et al.* [9]. The membranes were washed with TBS-Tween solution (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20). Then they were blocked with 0.3% BSA in TBS-Tween for 1 hr and treated with 10 μg/ml horseradish peroxidase-conjugated peanut agglutinin (HRP-PNA) for 1 hr. Control runs were made either by omitting the treatment with HRP-PNA or by treating the membranes with a mixture of HRP-PNA and 2.5 M galactose. The method of Adams [1] was used to detect HRP.

Preparation of antiserum

Antiserum was raised against isolated gp 105 by injection into rabbits. It was absorbed by glutaraldehyde-fixed pars convoluta (PC) tissue from the oviduct.

Immunoblot

Westernblotted membranes were blocked overnight in a phosphate-buffered saline solution (PBS, pH 7.2) containing 10% normal sheep serum and 4% BSA, treated for 1 hr with the antiserum diluted to 1/50 in PBS containing 4% BSA, and immersed for 1 hr with HRP-conjugated sheep antirabbit IgG according to the method of Smith [19]. A control was established by treating the membranes with non-immune rabbit serum in place of the antiserum. HRP was again detected by the Adams method.

Electron microscopy

Eggs were fixed overnight at 4°C in 2.5% glutaraldehyde in 100 mM cacodylate buffer (pH 7.4), rinsed in buffer, and postfixed for 3 hr in similarly buffered 1% OsO_4. The specimens were dehydrated in acetone and embedded in Quetol 812 (Nisshin EM Co.). Ultrathin sections were stained with uranyl acetate and lead citrate and viewed with a JEOL JEM-100SX electron microscope.

Immunoelectron microscopy

Glutaraldehyde-fixed eggs were dehydrated in ethanol and embedded in Lowicryl K4M (Sigma) at −20°C according to the manufacturer's instructions. Thin sections were mounted on collodion-coated nickel grids. The sections were blocked for 10 min with 0.5% BSA in PBS and washed with PBS. They were then incubated for 1 hr at room temperature with a 1/2,000 solution of antiserum. The sections were subsequently washed with PBS and treated for 1 hr with a gold-conjugated goat antiserum against rabbit IgG (E-Y Lab.). Then they were washed with PBS and with distilled water and stained with uranyl acetate and lead citrate. After staining, the sections were dried and coated with carbon vapor. Control sections were treated with a rabbit non-immune serum and then gold-conjugated goat antiserum. There was no significant labeling on the control sections.

Electron microscopic detection of carbohydrates

Ultrastructural localization of carbohydrates was detected by the periodic acid-chromic acid-silver methenamine (PA-CrA-Silver) method according to Rambourg *et al.* [17]. Glutaraldehyde-fixed specimens were dehydrated and embedded in Quetol 812. Thin sections were placed on uncoated stainless-steel grids for staining with PA-CrA-Silver. Control runs were made by treating the sections with silver methenamine without previous oxidation.

Assay of interaction of cortical granule lectins with gp 105

Westernblotted membranes were blocked overnight in DB solution containing 1% BSA and treated for 3 hr with gold-conjugated cortical granule lectins in DB solution containing 1% BSA. Controls were established by treating membranes with the gold-conjugated lectins plus either galactose (1 M) or EDTA (1 mM). The membranes were then processed for silver enhancement with a silver enhancing kit (Bio-Rad).

RESULTS

Isolation of gp 105 from the fertilization layer

The fertilization envelope (FE) consists of a vitelline envelope (VE*) and a fertilization (F) layer in *Xenopus* (Fig. 1A). The F layer was exposed by removal of the jelly with

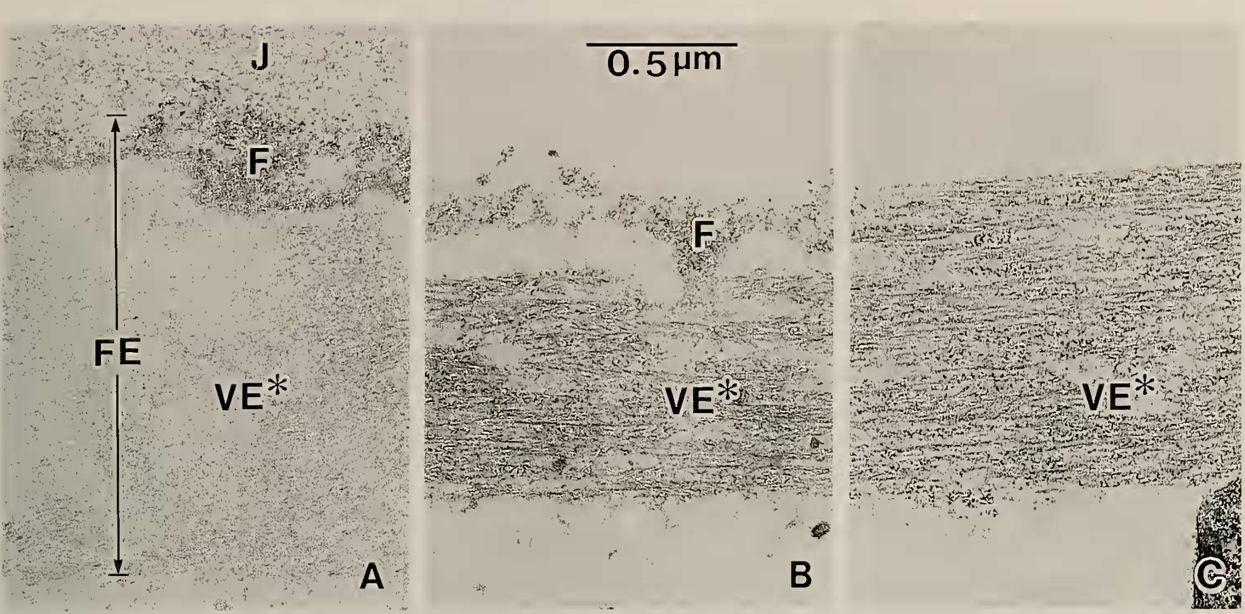


Fig. 1. Electron micrographs showing cross sections of egg envelopes of activated eggs (A), of envelopes whose jelly was removed with 20 mM DTT (B), and of an envelope whose fertilization (F) layer was removed with 5 mM EDTA (C). FE, fertilization envelope; VE*, modified vitelline envelope in the FE.

DTT (Fig. 1B) and was collected after solubilization with EDTA (Fig. 1C). Although DTT may have affected the morphology of the envelopes, as the F layer was loosened from the VE* because of DTT's low salinity, at this stage of investigation, clearer separation of the jelly and the F layer was desirable. The VE* appeared to decrease in width after jelly removal.

Electrophoretic profiles of FEs were compared with those of VE*s to find the constituents of the F layer (Fig. 2). A glycoprotein of 105 kDa (gp 105) appeared with Coomassie blue staining in the profile of the FE but not of the VE* (Fig. 2A), whereas no difference was observed with PAS staining (Fig. 2B). It was also confirmed that gp 105 is not a constituent of the jelly layers (Fig. 2, lane 3). Thus gp 105 is a constituent of the F layer. Although it was expected that 39–46-kDa protein bands of cortical granule lectins [29] would be present in the profiles of FE, the present study failed to show any lectin bands. The amount of the lectins contained in the FE loaded may have been subminimal for detection and/or they may have been hidden by relatively large amounts of gp 41.

Fig. 3 shows SDS-PAGE profiles of preparations at each step of the isolation procedure. Crude F layer extract (lane 1) was centrifuged at 7,000×*g* for 30 min; the pellet (lane 2) was suspended once in a 5 mM EDTA-20 mM DTT solution, freeze-thawed and reprecipitated by centrifugation at 1,000×*g* for 15 min. This 1,000×*g* precipitate (lane 3) contained glycoproteins of 105, 100, 30 and 29 kDa. The 105-kDa band in lane 3 was sliced out and the glycoprotein extracted electrophoretically. The extract (lane 4) gave a single band of gp 105 after reelectrophoresis.

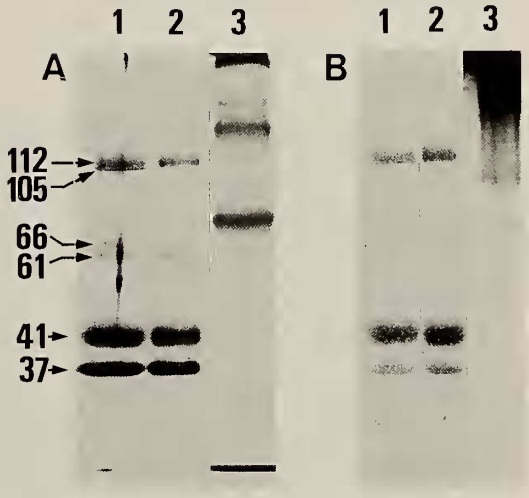


Fig. 2. SDS-PAGE of FE (5 μg, lane 1), VE* (5 μg, lane 2) and jelly (5 μg, lane 3) stained with Coomassie blue (A) or PAS (B). A 105-kDa glycoprotein (gp 105) can be seen only in the FE stained with Coomassie blue. In this and subsequent figures the molecular weights are indicated on the left in kilodaltons.

Immunoblotting demonstration of gp 105 in F layer

Figure 4A shows immunoblots of the 1,000×*g* precipitate (lane 1), FE (lane 2), VE* (lane 3) and jelly (lane 4). The membranes were treated with an antiserum raised against gp 105. There was significant staining on gp 105 and gp 100 in the 1,000×*g* precipitate of the F layer extract and on gp 105 in the FE but no specific staining on the VE* and the jelly. The degree of stainability on gp 37 of the FE and VE* was the same as that on the control (Fig. 4B); thus the staining on gp 37 was not specific.

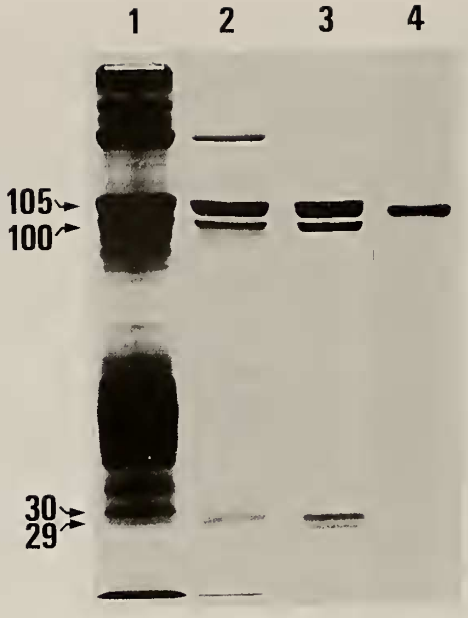


Fig. 3. SDS-PAGE of F layer extract at each step of the isolation procedure. Lane 1, crude extract (20 μg); lane 2, 7,000×g precipitate (15 μg) of the crude extract; lane 3, 1,000×g precipitate (15 μg) of the 7,000×g precipitates after suspension in a 5 mM EDTA-20 mM DTT solution and centrifugation; lane 4, gp 105 (5 μg) extracted from the lane 3 precipitate.

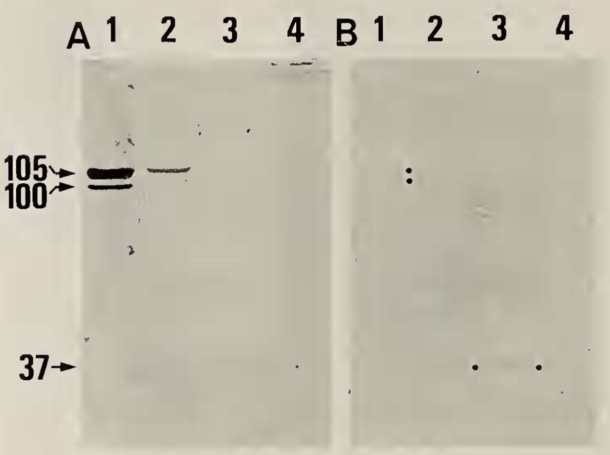


Fig. 4. Immunoblot analysis of a 1,000×g precipitate of F layer extract (10 μg, lane 1), FE (20 μg, lane 2), VE* (20 μg, lane 3) and jelly (10 μg, lane 4). Membrane A was treated with an antiserum to gp 105. There was staining on gp 105 and gp 100 in the F layer extract and gp 105 in the FE. Membrane B was treated with non-immune serum and shows faint staining on gp 105, gp 100 and gp 37 (to left of dots).

The experiments just discussed showed that gp 105 is a component of the FE but not its location. To localize gp 105 in the FE, the same immunoblotting procedure as above was performed on FEs obtained from embryos at stages 22, 26, 28 and from the 28+15 hr culture. gp 37 staining was used as a control for protein loading. The stainability on gp 105 increased during the hatching period (Fig. 5), which indicates that the relative amount of gp 105 gradually increased in the FEs during the hatching process. Since previous ultrastructural observations showed that the F layer remained substantially unaffected even after the VE* portion of the FE was completely digested by the hatching enzyme [32], it seems that gp 105 resides in the F layer.

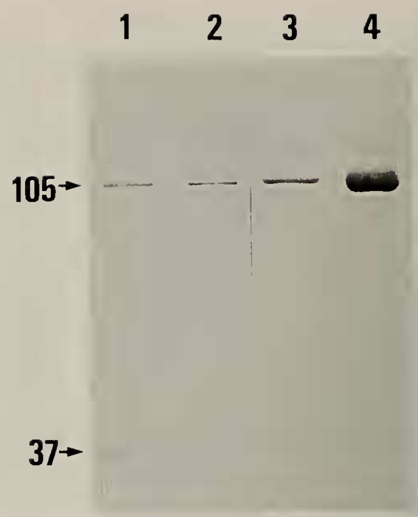


Fig. 5. Immunoblot analysis of FEs from embryos at various stages showing increased amounts of gp 105 as development proceeds. The amounts of FEs loaded in each lane were adjusted such that the amounts of gp 37 appeared approximately the same, in order to provide a control. Lane 1, stage 22; lane 2, stage 26; lane 3, stage 28; lane 4, stage 28+15 hr culture, as described in MATERIALS AND METHODS.

Immunoelectron microscopical localization of gp 105 in eggs

Sections of eggs obtained from various parts of oviducts were treated with antiserum against gp 105 and then with gold-conjugated goat antiserum, followed by electron microscope observation. Gold particles were absent in eggs obtained from the pars recta 1 (PR1)(Fig. 6A). In PR2 eggs they were present on the layer covering the outer surface of the vitelline envelope (VE), that is, the pre-fertilization (PF) layer (Fig. 6B). A gold-labeled PF layer lay between the VE and the innermost jelly layer in eggs obtained from the pars convoluta 1 (PC1; Fig. 6C), and it occasionally invaginated deeply into the VE in uterine eggs (Fig. 6D). The PF layer appeared to be compressed by the jelly layer. The jelly layer was substantially free of the gold labeling. These observations indicate that gp 105 resides in the PF layer of oviductal eggs which have passed through the PR2.

Figure 7 shows a section of activated eggs treated with the same two antisera as above. The F layer could be divided into two areas, a condensed region immediately adjacent to the outer margin of the VE* and a dispersed region peripheral to the condensed one [5, 23]. The gold particles were limited to the dispersed region.

Biochemical and ultrastructural demonstration of carbohydrates

Since carbohydrate residues are always found with ligand molecules of lectins, they were tested for in the gp 105. PAS

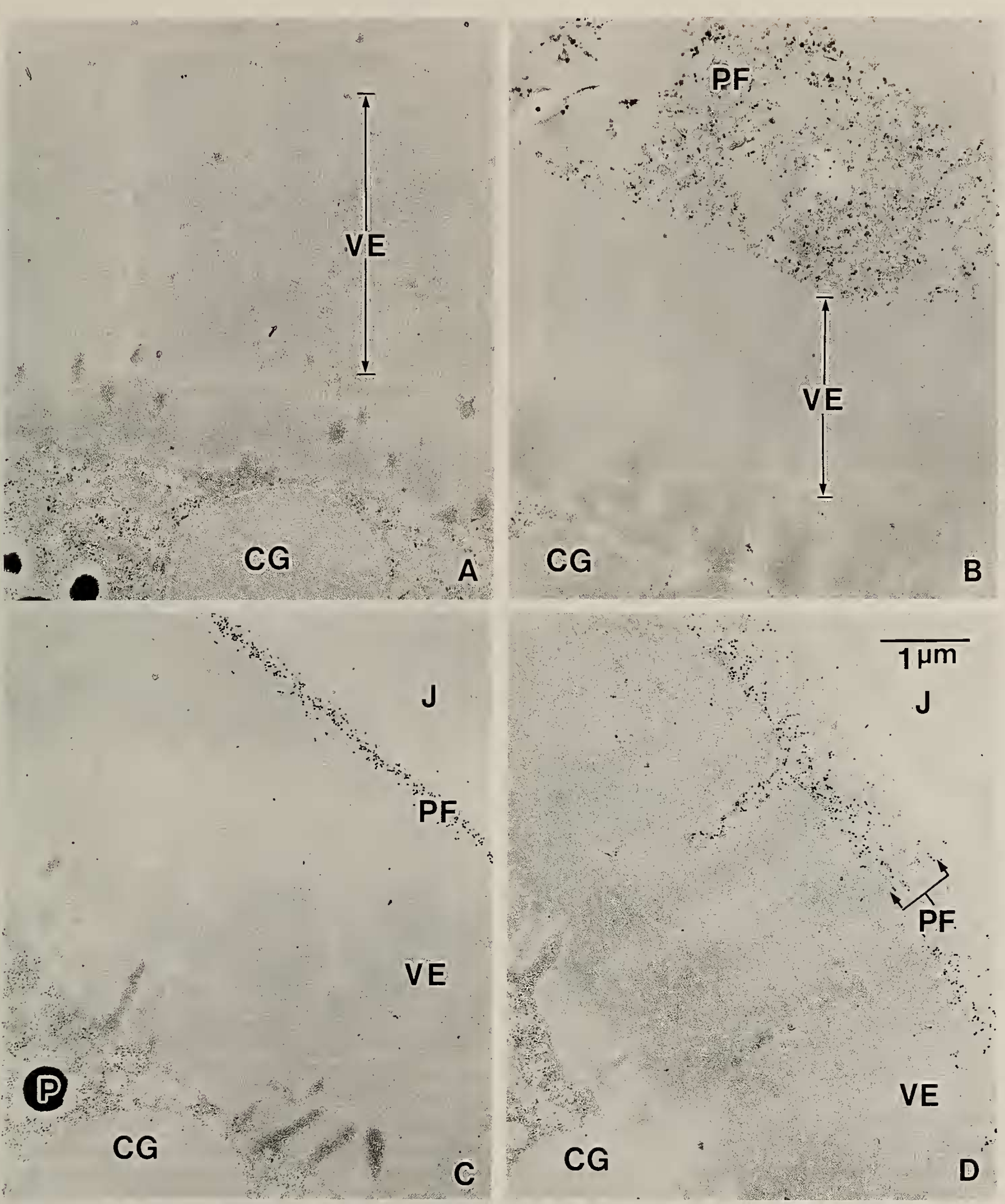


FIG. 6. Immunoelectron micrographs of sections of eggs from the pars recta 1 (A), pars recta 2 (B), pars convoluta 1 (C), and the uterus (D) of the oviduct. Gold particles indicating the location of gp 105 reside in the pre-fertilization (PF) layer adhering to the outer surface of the vitelline envelope (VE) in pars recta 2 eggs and between the VE and the jelly (J) layer in pars convoluta 1 eggs. Occasionally they invaginate into the VE in uterine eggs. CG, cortical granule; P, pigment geranule.

staining on gels of SDS-PAGE did not mark gp 105. (See Fig. 2B again). However, HRP-PNA stained gp 105 and gp 100 in the 1,000×g precipitate of F layer extract and gp 105, gp 66/61 and gp 37 in the FE (Fig. 8A, C). Galactose significantly decreased the staining (Fig. 8B). These results suggest that gp 105 possesses, although in very small amounts, carbohydrates of a galactoside nature.

Sections of oviductal or activated eggs were also treated

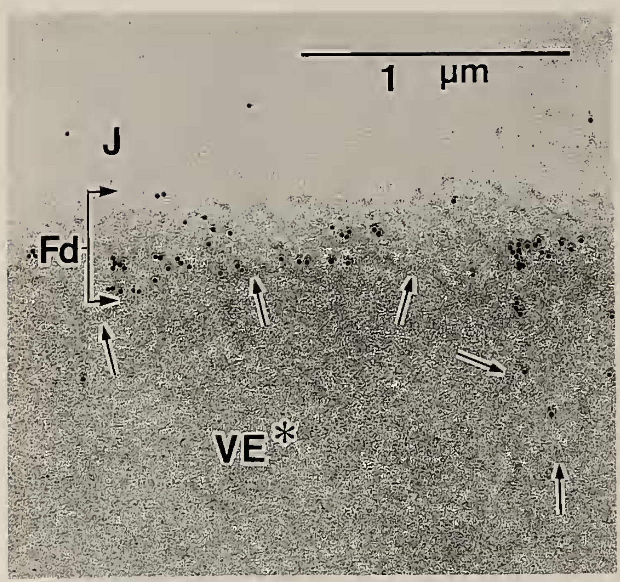


Fig. 7. Immunoelectron micrograph of a section of activated egg. Gold particles are present on the dispersed region of the fertilization (Fd) layer but absent from its condensed regions (arrows). J, jelly layer; VE*, modified vitelline envelope.

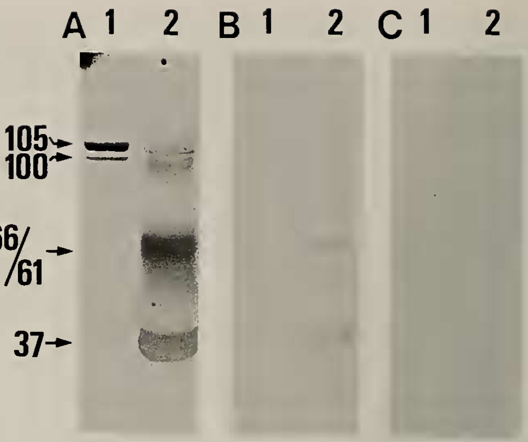
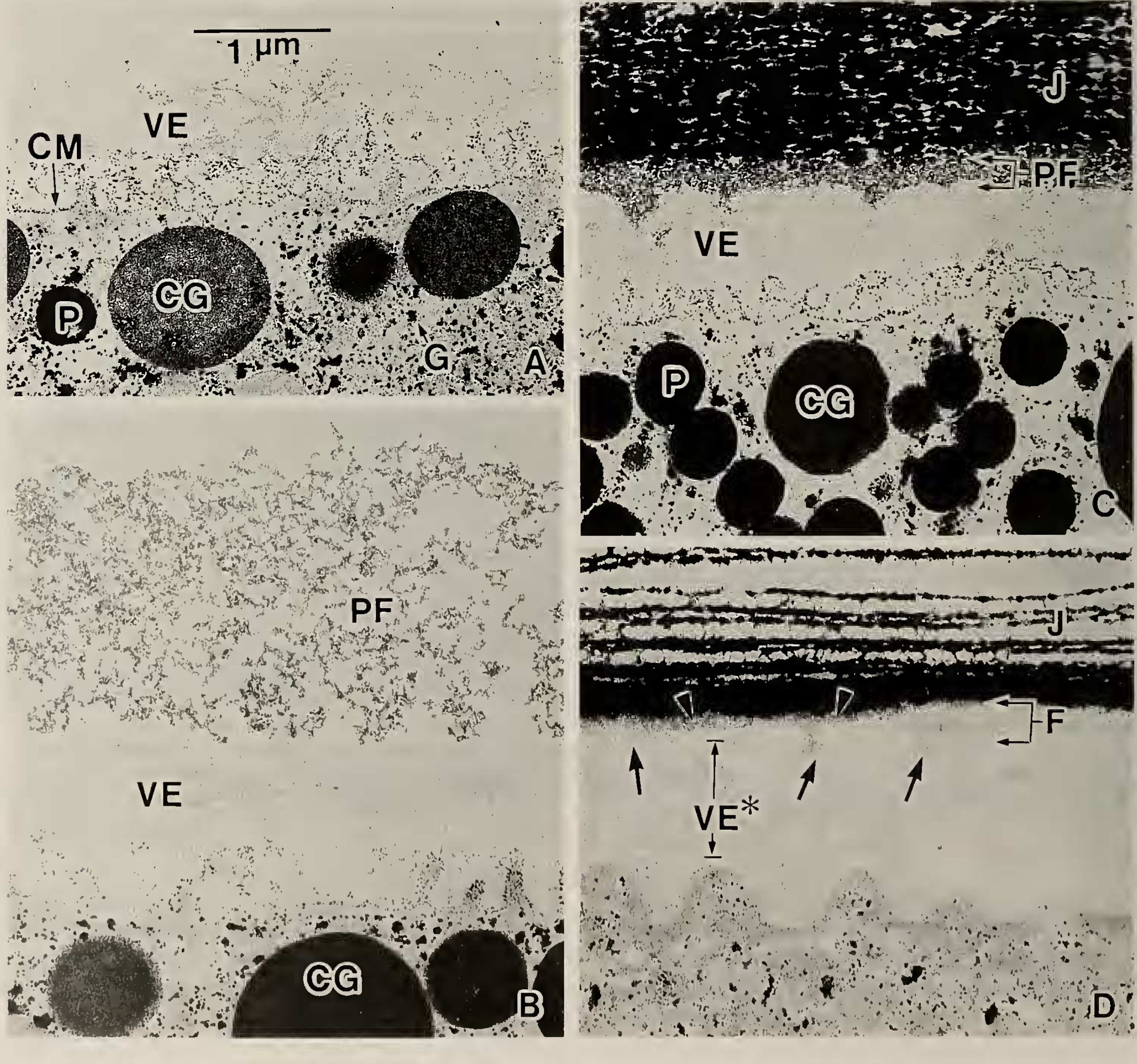


Fig. 8. Detection of galactosides on westernblots of a $1{,}000 \times g$ precipitate of the F layer extract (10 μg, lane 1) and of FE (30 μg, lane 2) by treatment with HRP-PNA (A), HRP-PNA plus galactose (B) or no treatment (C). The gp 105 and gp 100 in the F layer extract and the gp 105, gp 66/61 and gp 37 in the FE are stained (A). Galactose significantly decreases the staining (B).



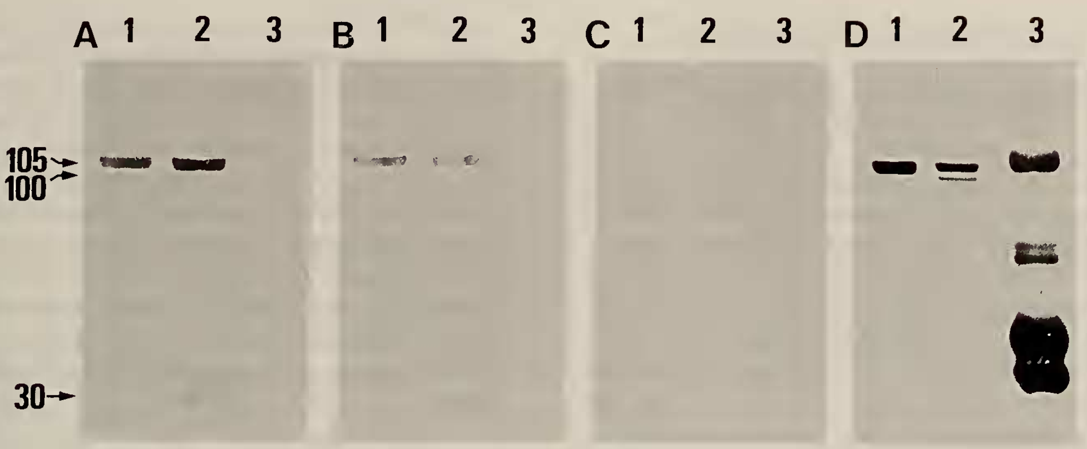


Fig. 10. Interaction of gp 105 with cortical granule lectins. Westernblots of isolated gp 105 (10 μg, lane 1), of a 1,000×g precipitate of the F layer extract (10 μg, lane 2) and FE (30 μg, lane 3) were treated with gold-conjugated lectins alone (approximately 20 μg/ml lectins; A) or with gold-conjugated lectins plus either galactose (1 M; B) or EDTA (1 mM; C). Blotting membranes were further treated with a silver enhancing kit. D, membrane stained with Coomassie blue.

according to the PA-CrA-Silver method for detecting carbohydrates (Fig. 9). In PR1 eggs (Fig. 9A), silver precipitates indicating the location of carbohydrates were present on the cell membrane, cortical granules and glycogen granules. The VEs of these eggs were not stained by the silver, but the PF layer in PR2 eggs (Fig. 9B) and uterine eggs (Fig. 9C) were. The jelly layer in uterine eggs was also stained, but a difference in stainability between the PF layer and the jelly layer is evident, since the silver precipitates on the PF layer are fine whereas those on the jelly layer are coarse and more tightly packed (Fig. 9C). In activated eggs (Fig. 9D), the dispersed region of the F layer was stained by the silver but its condensed region not.

Interaction of gp 105 with cortical granule lectins

Figure 10 shows westernblots of the isolated gp 105, a 1,000×*g* precipitate of F layer extract and FE. Gold-conjugated cortical granule lectins stained gp 105 in the first two samples (Fig. 10A). The staining on gp 105 in FE was weak, which may be due to a relatively low amount of gp 105 in FE. gp 100 and gp 30 of the 1,000×*g* precipitate were also stained. Galactose and EDTA, which are inhibitors of lectins [16, 28, 29], decreased the staining (Fig. 10B, C). Such results constitute evidence of the occurrence of a lectin-ligand reaction between gp 105 and the cortical granule lectins, thus indicating gp 105 is a ligand molecule of the lectins.

DISCUSSION

gp 105, a natural ligand to cortical granule lectins

In *Xenopus* the F layer becomes apparent after fertilization as an electron-dense layer between the VE* and the innermost jelly layer [25, 31]. Involvement of cortical granule lectins in the reaction forming the F layer was suggested by Wyrick *et al.* [25] and the localization of the lectins in the F layer was demonstrated by immunoelectron microscopy [30]. However, the identity of the ligand molecule binding to the lectins has been disputed [25, 31].

In the present study gp 105 was isolated from an extract of the F layer. On immunoblot and immunohistochemical test evidence, this glycoprotein is apparently not a component of the jelly. That gp 105 is a constituent of the F layer was suggested by the increase in the relative amount of gp 105 in FEs during the hatching process, an increase which coincides well with the previous ultrastructural finding that the F layer remained substantially unaffected even after the VE* portion of the FE was completely digested [32]. Gold particles indicating the location of gp 105 by immunoelectron microscopy were present on the PF layer of uterine eggs, distributed in the same area as that comprising the F layer of fertilized eggs according to Grey *et al.*[5]. Furthermore, binding of gold-conjugated cortical granule lectins to gp 105 on westernblotted membranes demonstrated that lectin-ligand reactions occur. The PF layer is the first extracellular matrix outside the VE met by cortical granule lectins as they emanate from the egg surface and extrude through the VE

Fig. 9. Ultrastructural localization of carbohydrates by application of the PA-CrA-Silver method to sections of egg from the pars recta 1 (A), pars recta 2 (B), uterus (C) and uterine egg after activation (D). Silver particles appear on both the pre-fertilization (PF) layer (B and C) and the jelly (J) layer (C and D), but those on the PF layer are fine whereas those on the J layer are coarse and more tightly packed. In activated egg (D), the dispersed region of the fertilization (F) layer is stained by the silver (arrowheads) but the condensed region of the F layer (which is visible as a line marked by the arrows) is not. CG, cortical granule; CM, cell membrane; G, glycogen granule; P, pigment granule; VE, vitelline envelope; VE*, VE of activated egg.

[22]. Then the natural ligand of the lectins seems to be the gp 105 in the PF layer.

Resistance to treatment by reducing reagents such as DTT is one of the characteristics of the F layer [23]. The PF layer, however, was dissolved in conjunction with the jelly when treated with mercaptoethanol (see, Wolf *et al.* [24]). A DTT-stable layer could be produced by activating PR2 eggs whose external surface was covered by the PF layer [31] or by treating them with lectins [28]. The present study demonstrated diminution in both the antigenicity of gp 105 and its stainability by the PA-CrA-Silver method at the time of change from PF to F layer. This diminution is understandable if the respective reactive sites on gp 105 are being hidden by added cortical granule lectins, as discussed later. Differential response to DTT between the PF layer and the F layer can be understood along the same lines.

The present study suggested that gp 105 is supplied to eggs at the PR2 of the oviduct. Previous studies have shown that an antiserum against secretory granules of the PR2 stained the PF layer [26, 31], suggesting a PR2 origin for PF layer substances. There are two types of secretory cells in the PR2 and PC1, respectively [27]. An intact antiserum raised against gp 105 in the present study stained not only both types of secretory cells in the PR2 but also one type of secretory cell at the ridge in the PC1 (unpublished). However, when absorbed by the PC1, the antiserum completely lost its ability to stain the cells of either the PC1 or PR2 (unpublished) but retained the ability to stain gp 105. The antiserum may recognize an epitope which is newly produced by an interaction between substances secreted by cells in the PR2.

The gp 100 in F layer extract exhibited the same antigenicity, HRP-PNA stainability and gold-conjugated cortical granule lectin binding as gp 105. It did not appear in the FEs but only in the extract. Since an antiserum raised against gp 105 and absorbed by both the jelly and ovarian homogenates still retained the ability to bind with gp 100 (unpublished), gp 100 is not a contaminant from the jelly, VE, or cellular components of the eggs. gp 100 may be a degraded form of gp 105, since it did not appear when the entire isolation procedure was performed in the presence of the protease inhibitors PMSF (1 mM) and aprotinin (1 μg/ml) (unpublished). Possible agents of degradation are trypsin-like and chymotrypsin-like enzymes [12, 13], both of which are released extracellularly from their binding sites after activation. The gp 30 in the F-layer extract also exhibited gold-conjugated cortical granule lectin binding but did not show HRP-PNA stainability nor antigenicity like gp 105. Thus gp 30 is different from gp 105.

The SDS-PAGE profiles of FE in Figure 2 do not show all of the substances seen in those of Gerton and Hedrick [4]. Proteins of 120 kDa and 57 kDa do not appear in this study. The absence of 120-kDa protein is probably due to the difference in the amount of FE loaded, 5 μg in the present study but 25 μg in Gerton and Hedrick's. gp 105 migrates on SDS-PAGE very close to gp 112 and they fuse with each other when there are large amounts of FE, so that in this study smaller amounts of FE than usual were chosen in order to separate the two glycoproteins. The 57-kDa protein also failed to appear in this study even though 30 μg FEs were loaded, as seen in Figure 10D. The reason for its absence is not clear yet. It is possible that the 57-kDa protein may have been removed from the FEs during the dejellying process in this study. To clearly separate the jelly from the FEs, this study exploited a Ca-free, 0.05 DB solution (a medium containing reducing agents), whereas Gerton and Hedrick adopted a full-strength DB solution. Further studies are needed to explain why the 57-kDa protein did not appear.

Carbohydrates in gp 105

Carbohydrate residues must be demonstrated in order to claim that a particular substance is in fact a ligand. Carbohydrates were detected histochemically in the PF layer and biochemically by the HRP-PNA method in gp 105. The PNA shares carbohydrate-specificity for galactosides with the cortical granule lectins [16, 28, 29]. Apparently, however, there is very little carbohydrate in gp 105, since it was not stained with the PAS method.

There seems to be a discrepancy with respect to histochemical results: PA-CrA-Silver staining, which detects carbohydrates ultrastructurally, stained cortical granules and the PF layer in unfertilized eggs but did not stain the F layer in activated eggs. gp 105 is glycosylated, as mentioned above, and so are cortical granule lectins [16]. With present knowledge, it is difficult to explain the failure in histochemical detection of F-layer carbohydrates, since they are produced by interactions of two biochemically defined glycoproteins. It seems possible that carbohydrate moieties of gp 105 are being bound by lectins in such a way or at such binding sites that the carbohydrate is unavailable for staining in the condensed region of the F layer (see Fig. 9). This region probably corresponds to the area of increased electron density under conventional heavy metal staining [5, 25, 31], which suggests the presence of some additional substance in the F layer, namely, lectins. Taken together, the increase in electron density and the absence of PA-CrA-Silver staining indicates that some reaction has occurred during fertilization; the next studies need to examine exactly how carbohydrate moieties of the cortical granule lectins behave at the time of binding with gp 105.

Ligand molecules in the jelly layer

Since cortical granule lectins can react with the jelly, the jelly layer has been proposed as the site of ligand molecules [25]. Using immunoelectrophoretic analyses, Birr and Hedrick [2] observed three jelly coat ligands bound by cortical granule lectins; two of the three (L-1 and L-2) were sulfated and one (L-3) not. Since L-3 was present in low concentration relative to other components in total jelly solutions and it cross-reacted with anti-envelope sera but not with anti-total jelly sera, they speculated that L-3 is a component of the PF layer. However, neither association of L-3 with the PF layer nor its molecular weight has been established yet. It has already been shown that in PF layer-depleted eggs, secreted lectins produced an electron-dense layer in the space between the outer surface of the VE* and the inner surface of the jelly layer but were dissolved together with the jelly when treated with DTT [31], suggesting that secreted lectins reacted with the jelly at the innermost surface of its layer but did not penetrate deeply into the jelly layer, and that most of the lectins accumulated in the space. Thus L-1 and L-2 (and perhaps L-3), associated with the innermost jelly coat layer, may be ligands to the cortical granule lectins and seem certain to have a role in natural fertilization. The relationship of these substances to gp 105 might warrant investigation.

The jelly layer may act as a block to outward diffusion of cortical granule lectins. Limited distribution of secreted lectins was demonstrated in activated eggs immunoelectron microscopically [30]: gold particles indicating the locations of the lectins were present in the perivitelline space, on the VE*, and on the F layer, but not on the jelly. Previous observations on morphological and biochemical changes in the FEs suggested that the F layer is resistant to the hatching enzyme secreted by hatching embryos [32]. A thick F layer might be disadvantageous for embryos to break through. As shown in the present study, the PF layer is compressed by externally loaded jelly layers during jelly deposition around the eggs. The jelly layer may minimize the space in which the F layer will be formed but, by blocking dispersing lectins at its innermost surface, guarantee sufficient lectin-gp 105 interactions for production of a polyspermy block.

The present study claims that the F layer is formed by the interaction of gp 105 with cortical granule lectins, but it does not preclude other as yet unrecognized elements of the F layer from candidacy for ligand molecule to the lectins. In particular, immunoelectrophoretically identified ligands of the jelly noted by Birr and Hedrick [2] may also be found in the F layer, for the jelly seems to participate in F layer formation, as discussed above, and the boundary between the jelly layer and F layer is not absolute. That multiple types of secretory cells are involved in producing the extracellular matrix of eggs [27] also suggests the existence of multiple ligand molecules in the F layer. Thus further study is needed to explore other ligand molecules than gp 105 in this layer.

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